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# Isolation and Characterization of Integron-Containing Bacteria without Antibiotic Selection

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**The emergence of antibiotic resistance among pathogenic and commensal bacteria has become a serious problem worldwide. The use and overuse of antibiotics in a number of settings are contributing to the development of antibiotic-resistant microorganisms. The class 1 and 2 integrase genes (*intI1* and *intI2*, respectively) were identified in mixed bacterial cultures enriched from bovine feces by growth in buffered peptone water (BPW) followed by integrase-specific PCR. Integrase-positive bacterial colonies from the enrichment cultures were then isolated by using hydrophobic grid membrane filters and integrase-specific gene probes. Bacterial clones isolated by this technique were then confirmed to carry integrons by further testing by PCR and DNA sequencing. Integron-associated antibiotic resistance genes were detected in bacteria such as *Escherichia coli*, *Aeromonas* spp., *Proteus* spp., *Morganella morganii*, *Shewanella* spp., and urea-positive *Providencia stuartii* isolates from bovine fecal samples without the use of selective enrichment media containing antibiotics. Streptomycin and trimethoprim resistance were commonly associated with integrons. The advantages conferred by this methodology are that a wide variety of integron-containing bacteria may be simultaneously cultured in BPW enrichments and culture biases due to antibiotic selection can be avoided. Rapid and efficient identification, isolation, and characterization of antibiotic resistance-associated integrons are possible by this protocol. These methods will facilitate greater understanding of the factors that contribute to the presence and transfer of integron-associated antibiotic resistance genes in bacterial isolates from red meat production animals.**

Bacteria are able to inherit antibiotic resistance genes to provide protection against most antibiotics. The dissemination of antibiotic resistance genes by horizontal gene transfer has led to the rapid emergence of antibiotic resistance among bacteria (13). Extensive studies have shown that mobile genetic elements such as plasmids and transposons are able to facilitate the spread of genetic material between species or genera of bacteria. In the 1980s, genetic elements termed integrons were identified on these mobile elements (20). Integrons are gene-capture and expression systems characterized by the presence of an *intI* gene encoding an integrase, a recombination site (*attI*), and a promoter (13). Integrons are able to capture gene cassettes from the environment and incorporate them by using site-specific recombination. To date, at least eight classes of integrons have been described (10). Each class is distinguished by differences in the sequences of the integrase genes. Integron classes 1 and 2 contain antibiotic resistance gene cassettes and have been the focus of numerous and widespread studies (5, 7, 12). Class 4 integrons have been termed superintegrons and are found on the small chromosome of *Vibrio cholerae*. Superintegrons harbor hundreds of gene cassettes which encode adaptations that extend beyond antibiotic resistance and pathogenicity (14). The remaining classes of integrons may also contain antibiotic resistance gene cassettes, but their worldwide prevalence remains low (2, 10). Integrons play a major role in the dissemination of antibiotic resistance genes in gram-negative bacteria and are commonly associated with members of the family *Enterobacteriaceae* (4).

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Antibiotic use in clinical and nonclinical settings plays an important part in the development of antibiotic-resistant bacteria throughout the world (3). The use of antibiotics as prophylactics and growth promoters in food-producing animals and its effect on the development of antibiotic-resistant bacteria is one area that has become topical in recent times. Of particular concern is the potential for antibiotic-resistant bacteria or the antibiotic resistance genes carried by these strains to enter the food chain and, consequently, to be passed on to humans (19). Evaluation of integrons containing antibiotic resistance genes provides a means to rapidly assess the potential reservoir of one form of antibiotic resistance that may be present in bacterial populations associated with meat production animals. Methods capable of detecting, isolating, and characterizing integron-containing antibiotic-resistant bacteria may allow the assessment of this form of antibiotic resistance gene transfer between bacterial hosts.

The isolation of bacteria expressing resistance to antibiotics has traditionally relied on the selection of bacteria capable of growth in liquid or on solid media containing specific antibiotics (22). If the medium chosen is specific for the growth of a limited group of bacteria or a specific bacterial genus, the selection of antibiotic-resistant isolates may be relatively routine. However, if the medium supports the growth of a wide range of bacteria, it can be anticipated that a corresponding wide range of bacteria that apparently express antibiotic resistance will also be propagated. Since different bacterial genera and species exhibit various levels of intrinsic antibiotic resistance, it is likely that some bacterial isolates will express bona fide antibiotic resistance, conferred by an active antibiotic resistance mechanism, while others will show inherent resistance due to factors such as decreased uptake of particular antibiot-

ics. In such cases, substantial efforts will be necessary to discriminate between isolates which show inherent resistance and those which have active resistance mechanisms gained through antibiotic resistance genes. The development of methods which may facilitate the identification of antibiotic-resistant bacteria, independent of their selection on media supplemented with an antibiotic(s), will greatly expedite both the efficiency of isolation and assessment of the range of antibiotic resistance genes carried by antibiotic-resistant isolates.

To date no protocols that enable the isolation of integron-containing antibiotic-resistant bacteria from mixed populations have been described. The study of integron-borne antibiotic resistance in members of the family *Enterobacteriaceae* has been dominated by clinic-based research and has centered on the phenotypic and genotypic screening of bacteria isolated from symptomatic patients (8, 18, 23). While this approach is useful for monitoring the development of antibiotic resistance within clinically important bacteria, it almost certainly underestimates the variety of integron-associated antibiotic resistance genes that exist and hence the development of antibiotic-resistant bacteria. In the present study we describe a protocol that we developed. The protocol is capable of identifying, isolating, and characterizing integron-containing antibiotic-resistant bacteria from animal feces but is not dependent on their expression of antibiotic resistance genes.

## MATERIALS AND METHODS

**Bacterial strains.** Three control strains of *Escherichia coli* carrying class 1 integron-containing plasmids R388 and R46 and class 2 integron-containing transposon Tn*7*, respectively, were kindly provided by Hatch Stokes (Macquarie University). Plasmid R388 carries a class 1 integron with two gene cassettes, *dfrB*2 (which encodes trimethoprim resistance) and *orfA*. Plasmid R46 also carries a class 1 integron with four gene cassettes. R46 has two identical copies of *oxa2* (which encodes ampicillin resistance) and one copy each of *aadA1* (which encodes spectinomycin and streptomycin resistance) and *orfD*. Transposon Tn*7* carries a class 2 integron with three gene cassettes, *drfA1* (which encodes trimethoprim resistance), *sat* (which encodes streptothricin resistance), and *aadA1* (which encodes spectinomycin and streptomycin resistance). The control strains were maintained on Luria-Bertani agar containing ampicillin or streptomycin at 100 μg/ml.

**Sample collection and processing.** Five fecal samples were collected from each of 10 herds of cattle over a 1-month period. All fecal samples were collected from cattle that had been held overnight at a local abattoir. Each herd of cattle originated from independent geographic locations separated by at least 50 km. A 10-g quantity of feces was placed in 90 ml of buffered peptone water (BPW; Oxoid, Basingstoke, England), treated with a stomacher for 1 min, and subsequently enriched for 6 h at 37°C without agitation. A 100-μl aliquot of enrichment was reenriched in 100 ml of BPW for a further 18 h at 37°C. DNA templates were prepared from the resulting enrichments by boiled cell lysis. Briefly, 1 ml of overnight enrichment was pelleted by centrifugation, resuspended in 200 μl of sterile distilled water, and boiled for 10 min. The boiled cell suspensions were centrifuged, and the resulting lysate was used for PCR.

**PCR detection of integrase genes.** Boiled cell lysates were tested by PCR for the presence of integrase genes *intI1* and *intI2* by using specific primers designed to amplify conserved regions of the respective genes (Table 1). Primers HS 463a and HS 464 were used to detect *intI1*-positive samples. Primers RB 201 and RB 202 were used to detect *intI2*-positive samples. The PCRs were performed in 25-μl reaction mixtures containing 2 μl of DNA, 1.0 μM each oligonucleotide primer, 200 μM deoxynucleoside triphosphates (Roche Diagnostics, Mannheim, Germany), 10 mM Tris-HCl, 1.5 mM $MgCl_2$, 50 mM KCl (Roche Diagnostics), and 1 U of *Taq* polymerase (Roche Diagnostics). PCR was performed for 30 cycles of denaturation at 94°C for 30 s, annealing at 65°C (*intI1*) and 62°C (*intI2*) for 30 s, and extension at 72°C for 45 s. The PCR products were visualized by ethidium bromide staining after agarose gel electrophoresis.

**Isolation of integron-containing bacteria.** Enrichments positive for *intI1* and/or *intI2* were serially diluted to $10^{-5.5}$ in 0.1% peptone–1% Tween 80. One milliliter of the dilution was filtered through a hydrophobic grid membrane filter (HGMF) by using a spread filter (Filtaflex, Almonte, Ontario, Canada) and a vacuum manifold. HGMFs were placed onto modified hemorrhagic colitis (mHC) agar (21), which did not include methylumbelliferyl glucuronide, and were incubated overnight at 37°C. Colonies grown overnight were replicated onto a fresh HGMF by use of an HGMF replicator-inoculator system (Filtaflex) and incubated as described above. The bacterial colonies on the original HGMF were lysed by the method of Nizetic et al. (11) after wetting of the membranes with a pretreatment solution for 30 min (24). The DNA was cross-linked to the HGMF by exposure to UV light for 5 min.

TABLE 1. Primers used for PCR detection of class 1 and class 2 integrases and associated gene cassette regions

| Primer | Sequence | Reference |
|---|---|---|
| RB 201 | 5′-GCAAACGCAAGCATTCATTA-3′ | This study[a] |
| RB 202 | 5′-ACGGATATGCGACAAAAAGG-3′ | This study[b] |
| RB 317 | 5′-GAACCTTGACCGAACGCAG-3′ | This study[c] |
| RB 320 | 5′-AGCTTAGTAAAGCCCTCGCTAG-3′ | This study[d] |
| Hep 74 | 5′-CGGGATCCCGGACGGCATGCACG ATTTGTA-3′ | White et al. (23) |
| Hep 51 | 5′-GATGCCATCGCAAGTACGAG-3′ | White et al. (23) |
| HS 463a | 5′-CTGGATTTCGATCACGGCACG-3′ | Hatch Stokes, personal communication |
| HS 464 | 5′-ACATGCGTGTAAATCATCGTCG-3′ | Hatch Stokes, personal communication |

[a] Nucleotides 11918 to 11937 of *intI2* (GenBank accession no. AP002527).
[b] Complement of nucleotides 12292 to 12311 of *intI2* (GenBank accession no. AP002527).
[c] Nucleotides 2250 to 2268 of *intI1* (GenBank accession no. M95287).
[d] Complement of nucleotides 5538 to 5559 of *qacEΔ1* (GenBank accession no. M95287).

Membranes containing cross-linked DNA were hybridized with digoxigenin (DIG)-labeled probes capable of detecting *intI1* or *intI2* generated with primers HS 463a and HS 464 (*intI1*-specific probe) and primers RB 210 and RB 202 (*intI2*-specific probe) and the PCR DIG labeling kit (Roche Diagnostics) according to the instructions of the manufacturer. Hybridization was carried out at 68°C by the methods outlined in the DIG System User's Guide to Filter Hybridization (Roche Diagnostics). Presumptive *intI1*- and *intI2*-positive colonies were picked from replicate HGMFs and streaked onto nutrient agar plates (Oxoid).

**Confirmation and preliminary characterization of integrons.** Presumptively integrase-positive isolates were confirmed to be integrase positive by PCR. All isolates containing *intI1* were tested for the presence of *intI2*, and likewise, all *intI2*-positive isolates were tested for the presence of *intI1*. Primers able to amplify the inserted gene cassette regions of class 1 and class 2 integrons were used to determine the sizes of the inserted cassette regions. Primers RB 317 and RB 320 were used to amplify the class 1 integron gene cassette region (Table 1). PCR cycling conditions for the class 1 integron gene cassette region PCR were 30 cycles of denaturation at 94°C for 30 s, annealing at 59°C for 30 s, and extension at 72°C for 3 min. The PCR of White et al. (23) was used to amplify the class 2 integron gene cassette region.

Purification of PCR products was achieved by isopropanol precipitation. Briefly, 45 μl of each PCR mixture was added to 180 μl of 75% isopropanol. The samples were incubated at room temperature for 15 min to allow precipitation of the PCR products. The samples were then placed in a microcentrifuge and spun at 17,000 × *g* for 20 min. The resulting supernatant was carefully aspirated, and then 250 μl of 75% isopropanol was added and the samples were centrifuged at 17,000 × *g* for 5 min. Following centrifugation, the supernatants were carefully aspirated and the resulting DNA pellets were dried at 90°C for 1 min. The pellets were finally resuspended in 60 μl of TE (Tris-EDTA) buffer (pH 8.0). Quantification of the DNA concentration of each sample was performed by using gel electrophoresis and undigested bacteriophage λ DNA quantification standards of 2, 4, 6, 8, 10, and 20 ng/μl (Gibco BRL, Rockville, Md.).

Sequencing reactions were performed by the use of BigDye terminator (version 2.0; Applied Biosystems) chemistry and a 9700 thermal cycler (Perkin-Elmer, Norwalk, Conn.). Each reaction mixture contained 8 μl of the BigDye terminator (version 2.0) ready reaction mixture and 3.2 pmol of primer in a 20-μl reaction mixture. The primers used for sequencing were the same as those used to generate the PCR amplicon. The PCR program for all sequencing reactions included initial denaturation at 94°C for 5 min, followed by 25 cycles of denaturation for 10 s at 96°C, primer annealing for 5 s at 50°C, and extension for 4 min at 60°C. The sequencing products were purified by the isopropanol precipitation

protocol outlined above and were transported on ice to the Australian Genome Research Facility (University of Queensland, Brisbane, Australia) for sequencing. The resulting sequences were analyzed by searching the GenBank database of the National Center for Biotechnology Information via the BLAST network service (1). Sequences were aligned and compared with the sequences in the GenBank database by using the ContigExpress component of the Vector NTI Suite (version 5.5; InforMax, Inc., Frederick, Md.).

**Isolate identification and antibiotic susceptibility testing.** Identification of integron-containing organisms and the MICs of antibiotics for the organisms were determined by using the VITEK Junior system (BioMerieux, Hazelwood, Mo.). VITEK cards for identification (GNI cards) and antibiotic susceptibility testing (GNS-424 cards) were inoculated and incubated according to the recommendations of the manufacturer. The following antibiotics were tested: amikacin, amoxicillin-clavulanic acid, ampicillin, cefotaxime, ceftazidime, cephalothin, ciprofloxacin, gentamicin, imipenem, meropenem, nitrofurantoin, norfloxacin, ticarcillin-clavulanic acid, tobramycin, trimethoprim-sulfamethoxazole, and trimethoprim. A test for the detection of extended-spectrum β-lactamases (ESBLs) was also carried out, and interpretation of the results was based on comparison of the reduction in growth caused by cefotaxime-clavulanate and ceftazidime-clavulanate and that caused by the cephalosporins alone. The outcome of the test was either ESBL positive or ESBL negative, as determined by the VITEK Junior apparatus. *E. coli* ATCC 25922 was used as a control organism.

## RESULTS

**Prevalence of integron-containing bacteria in bovine feces.** A total of 50 bovine fecal samples from 10 geographically independent cattle herds were tested for the presence of class 1 and class 2 integrases. Overall, 43 of 50 (86%) samples tested positive for the class 1 integrase and 47 of 50 (94%) were positive for the class 2 integrase. Analysis of each herd for the presence of the class 1 integrase identified six herds for which five of five samples were positive, two herds for which four of five samples were positive, and one herd each for which three of five and two of five samples were positive. The analysis of each herd for the presence of the class 2 integrase also identified eight herds for which five of five samples were positive, one herd for which four of five samples were positive, and another herd for which three of five samples were positive.

Isolates containing class 1 integrons were isolated from 25 of 50 (50%) samples, and isolates containing class 2 integrons were isolated from 14 of 50 (28%) samples. Nine samples had both a class 1 integron-containing organism and a class 2 integron-containing organism, and five samples contained more than one class 1 integron-containing organism. No isolates containing both class 1 and class 2 integrons were found.

**Isolate identification and antibiotic susceptibility testing.** All isolates confirmed to carry either a class 1 or a class 2 integron were identified to the species level with the VITEK Junior system. Class 1 integrons were found in *Aeromonas caviae*, *Aeromonas veronii* biovar sobria, and *E. coli*. Class 2 integrons were found in urea-positive *Providentia stuartii* isolates, *E. coli*, *Proteus vulgaris*, *Proteus mirabilis*, *Shewanella putrefaciens*, and *Morganella morganii*. Each isolate was given an Abr identifier number, and the antibiotic susceptibilities of the integron-containing isolates were determined by using the GNS-424 cards, which determine the susceptibilities of gram-negative bacteria. The antibiotic resistances of the strains tested are shown in Table 2.

**Characterization of inserted gene cassette regions of class 1 and class 2 integrons.** To validate the success of our approach to detecting bacterial isolates carrying integrons encoding antibiotic resistance, preliminary characterization of the inserted gene cassette regions was performed. The inserted gene cassette regions of class 1 and class 2 integrons were amplified by PCR with primers RB 317 and RB 320 for class 1 integron-containing organisms and primers Hep 74 and Hep 51 for class 2 integron-containing organisms (Table 1). The PCR amplicons produced by primers RB 317 and RB 320 ranged in size from 900 to 2.1 kb and included variable regions with the capacity to code for between one and three inserted gene cassettes. A few isolates did not produce an amplicon when they were tested for the presence of an inserted gene cassette region. This was probably due to the lack of a 3′ conserved segment in these organisms. The PCR amplicons produced by primers Hep 74 and Hep 51 were 2.2 kb and were the same size as the amplicon produced from the Tn*7* control strain. Urea-positive *P. stuartii* isolates that were *intI2* positive failed to produce an amplicon of any size when they were tested.

Partial direct sequencing of the gene cassette regions of 15 randomly selected class 1 integrons was performed to validate the preliminary evidence for the presence of antibiotic resistance gene cassettes. This approach determined the presence of open reading frames with identities to genes encoding dihydrofolate reductases (*dfr2D*, *dfrA1*, *dfrXII*, and *dfrV*), aminoglycoside adenyltransferases (*aadA1* and *aadA2*), and chloramphenicol acetyltransferase (*catB8* and *catB3*) (Table 2). Partial sequencing of class 2 integrons indicated the presence of sequences orthologous to the trio of antibiotic resistance genes (*drfA1*, *sat*, and *aadA1*) associated with Tn*7* (Table 2).

## DISCUSSION

The integron detection protocol developed in this study was validated with 50 bovine fecal samples collected from local abattoirs. PCR testing indicated the presence of *intI1* (86%) or *intI2* (94%) in fecal samples, while bacteria containing class 1 or class 2 integrons were isolated from 50 and 28% of the samples, respectively. The reasons for the reduced isolation rates were not clear; however, they may have been due in part to the use of mHC agar as the growth medium for the HGMFs. mHC agar is designed to encourage the growth of members of the family *Enterobacteriaceae* and inhibit the growth of nonenteric bacteria (21). It is possible that a subset of those samples that tested positive by PCR for the class 1 or 2 integrase may contain integron-containing bacteria that do not grow on the semiselective agar. Use of media permissive for the growth of a broader range of bacteria may result in an improved isolation rate.

Multiple-resistance phenotypes were observed among both class 1 and class 2 integron-containing isolates. A high correlation between the presence of integrons carrying dihydrofolate reductase and the phenotypic expression of resistance to trimethoprim was observed. Many isolates also showed resistance to antibiotics but did not possess the corresponding antibiotic resistance gene cassettes within the integrons characterized from such isolates. It is probable that such resistances are encoded by nonintegron elements.

The partial sequencing of the integron-associated antibiotic resistance cassettes identified open reading frames with identities to genes that encode resistance to trimethoprim, streptomycin and spectinomycin, streptothricin, and chloramphenicol. Orthologous dihydrofolate reductase and aminoglycoside adenyltransferase gene sequences were found in the majority

TABLE 2. Antimicrobial resistance profiles of integron-containing bacteria

| Isolate | Organism | Integrase | Resistance[a] | Putative gene cassette(s)[c] |
|---|---|---|---|---|
| Abr31b | *E. coli* | 1 | AMP SXT, TMP | *dfrXII-aadA2* |
| Abr32a | *A. caviae* | 1 | AMP | ND |
| Abr33a | *E. coli* | 1 | AMP SXT, TMP (CF)[b] | ND |
| Abr34b | *E. coli* | 1 | SXT, TMP | *dfrV* |
| Abr35a | *E. coli* | 1 | SXT, TMP | ND |
| Abr36a | *E. coli* | 1 | SXT, TMP | ND |
| Abr40b | *A. caviae* | 1 | AMP | ND |
| Abr41a | *A. caviae* | 1 | (AMP) | ND |
| Abr41b | *E. coli* | 1 | AMP | *aadA2* |
| Abr48a | *A. veronii* biovar sobria | 1 | SXT | *dfr2D-aadA1* |
| Abr49a | *A. caviae* | 1 | AMP | ND |
| Abr49b | *A. caviae* | 1 | SXT | ND |
| Abr50a | *A. caviae* | 1 | None | ND |
| Abr51b | *A. caviae* | 1 | None | *aadA2* |
| Abr48b | *A. caviae* | 1 | SXT | ND |
| Abr47b | *A. caviae* | 1 | None | ND |
| Abr30b | *E. coli* | 1 | SXT, TMP | *dfrXII-aadA2* |
| Abr27a | *E. coli* | 1 | AMP, SXT, TMP | *dfrXII-aadA2* |
| Abr29a | *E. coli* | 1 | AMP, SXT, TMP | *dfrXII-aadA2* |
| Abr28a | *E. coli* | 1 | AMP, SXT, TMP | ND |
| Abr30a | *A. caviae* | 1 | AMP | *aadA2* |
| Abr31a | *A. caviae* | 1 | AMP | *aadA2* |
| Abr37a | *A. veronii* biovar sobria | 1 | SXT | *dfr2d-catB3-aadA1* |
| Abr37b | *A. veronii* biovar sobria | 1 | SXT, (TCC, TOB) | *dfr2d-catB3-aadA1* |
| Abr38a | *E. coli* | 1 | AMC, AMP, CF, SXT, TMP, (TAZ) | ND |
| Abr39b | *A. caviae* | 1 | (AMP) | *catB8-aadA1* |
| Abr43b | *A. veronii* biovar sobria | 1 | None | *aadA2* |
| Abr44a | *A. caviae* | 1 | AMP, SXT | ND |
| Abr45a | *A. caviae* | 1 | AMP | ND |
| Abr46a | *A. caviae* | 1 | SXT, (AMP) | *aadA2* |
| Abr47a | *A. caviae* | 1 | AM, SXT | ND |
| Abr52a | *A. caviae* | 1 | None | ND |
| Abr54a | *E. coli* | 2 | None | ND |
| Abr55a | *P. stuartii*, urea positive | 2 | AMC, AMP, CF, FD | ND |
| Abr56a | *P. stuartii*, urea positive | 2 | AMC, AMP, CF, FD | ND |
| Abr57a | *P. stuartii*, urea positive | 2 | AMC, AMP, CF, FD | ND |
| Abr58a | *E. coli* | 2 | TMP | *dfrA1-sat-aadA1* |
| Abr59a | *E. coli* | 2 | TMP | *dfrA1-sat-aadA1* |
| Abr60a | *E. coli* | 2 | TMP | *dfrA1-sat-aadA1* |
| Abr60b | *E. coli* | 2 | SXT, TMP | *dfrA1-sat-aadA1* |
| Abr61a | *P. vulgaris* | 2 | AMC, AMP, CF, FD | *dfrA1-sat-aadA1* |
| Abr62a | *M. morganii* | 2 | AMC, AMP, CF, FD, TMP, (TAX, TAZ, IMI) | *dfrA1-sat-aadA1* |
| Abr62b | *E. coli* | 2 | SXT, TMP | *dfrA1-sat-aadA1* |
| Abr63a | *E. coli* | 2 | SXT, TMP | *dfrA1-sat-aadA1* |
| Abr64a | *P. stuartii*, urea positive | 2 | AMC, AMP, CF, FD | ND |
| Abr65a | *E. coli* | 2 | TMP | *dfrA1-sat-aadA1* |

[a] Antibiotic abbreviations: AMP, ampicillin; AMC, amoxicillin-clavulanic acid; CF, cephalothin; FD, nitrofurantoin; IMI, imipenem; SXT, trimethoprim-sulfamethoxazole; TAX, cefotaxime; TAZ, ceftazidime; TCC, ticarcillin-clavulanic acid; TMP, trimethoprim; TOB, tobramycin.

[b] Parentheses indicate intermediate resistance.

[c] ND, not determined. Putative gene cassettes were identified by partial nucleotide sequencing and comparison with the sequences in the GenBank database by use of the BLAST service (1).

of class 1 integron-containing organisms. *Aeromonas* isolates that carried class 1 integrons were most likely to carry a dihydrofolate reductase gene ortholog and a downstream aminoglycoside adenyltransferase gene ortholog. The same observation has been made for motile aeromonads isolated from Danish rainbow trout farms and clinical specimens from the United Kingdom (9, 17). The chloramphenicol acetyltransferase gene (*catB8*) ortholog, detected in an *A. caviae* isolate carrying a class 1 integron, has previously been found to be associated with class 1 integrons in *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* (GenBank accession nos. AF227506 and AF418284, respectively). The inserted gene cassette regions of the class 2 integrons detected in this study were partially sequenced and compared with the class 2 integron carried on Tn*7* (6). All such sequences from *E. coli*, *M. morganii*, and *P. vulgaris* were identical to the sequences of the genes carried on Tn*7*, and it is therefore highly likely that these isolates also contain Tn*7* or related transposons.

The gene cassette regions of a number of class 1 and 2 integron-containing organisms could not be amplified by PCR. These results may have occurred because the inserted gene cassette regions were too large to be amplified by conventional PCR techniques or such integrons may lack the 3′ conserved segment generally associated with this class of integron. The inability of the class 2 integron gene cassette-specific PCR to amplify a product from any of the urea-positive *P. stuartii*

isolates may have been due to the presence of a class 2 integron that contains the 3′ conserved segment of class 1 integrons similar to the one found in an *Acinetobacter baumannii* isolate (12). Of further interest was the fact that the class 2 integrase-containing urea-positive *P. stuartii* isolates did not show resistance to trimethoprim, while all previously described class 2 integrons have encoded resistance to trimethoprim. Taken together, the absence of trimethoprim resistance in urea-positive *P. stuartii* isolates and the absence of amplicons from the class 2 integron gene cassette-specific PCR suggest that the class 2 integrase genes of the urea-positive *P. stuartii* isolates are not part of Tn*7* or related genetic elements. Subsequent characterization may determine if the urea-positive *P. stuartii intI2* genes are associated with antibiotic resistance cassettes or, alternatively, are part of a novel superintegron structure, proposed as the progenitor of antibiotic resistance integrons (15, 16).

The purpose of this study was to develop and validate a protocol that could be used to identify, isolate, and characterize class 1 and class 2 integron-containing bacteria from environmental samples. Present methods for the isolation of antibiotic-resistant bacteria depend on the selection of such isolates on antibiotic-containing media. A significant advantage of the protocol developed in this work is that bacteria carrying integron-associated antibiotic resistance gene cassettes will now be able to be isolated without prior selection of the bacteria on antibiotic-containing culture media. This will provide an opportunity to examine antibiotic resistance genes that have not been detected on the basis of their selective expression and may facilitate the identification of antibiotic resistance genes that are silently carried by the host bacteria. While silently carried antibiotic resistance genes may provide no advantage in their immediate bacterial host, transfer to another host may then be followed by expression. Rapid and efficient identification, isolation, and characterization of antibiotic resistance integrons are possible by this protocol. This protocol may now be applied to facilitate greater understanding of the factors that contribute to the presence and transfer of integron-associated antibiotic resistance genes in bacterial isolates from diverse environmental and animal sources.

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